Cytochromes P450 in Benzene Metabolism and Involvement of Their Metabolites and Reactive Oxygen Species in Toxicity

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Cytochrome P450 (CYP) 2E1 was the most efficient CYP enzyme that oxidized benzene to soluble and covalently bound metabolites in rat and human liver microsomes. The covalent binding was due mostly to the formation of benzoquinone (BQ), the oxidation product of hydroquinone (HQ), and was inversely related to the formation of soluble metabolites. In rats, inhalation of benzene (4 mg/liter of air) caused a rapid destruction of CYP2B1 previously induced by phenobarbital. The ability of benzene metabolites to destroy liver microsomal CYP *in vitro* decreased in the order BQ > HQ > catechol > phenol. The destruction was reversed by ascorbate and diminished by α-tocopherol, suggesting that HQ was not toxic, whereas BQ and semiquinone radical (SQ) caused the effect. In the presence of nicotinamide adenine dinucleotide phosphate, reduced (NADPH) the microsomes did not oxidize HQ to BQ, while the formation of superoxide anion radical from both HQ and BQ was markedly quenched. Destruction of CYP *in vitro* caused by HQ or BQ was not mediated by hydroxyl radical formation or by lipid peroxidation. On the contrary, HQ and BQ inhibited NADPH-mediated lipid peroxidation. Ascorbate induced high levels of hydroxyl radical formation and lipid peroxidation, which were differentially affected by quinones, indicating different mechanisms. Despite reducing the toxicity of HQ and BQ, ascorbate appeared to induce its own toxicity, reflected in high levels of lipid peroxidation. Iron redox cycling played a significant role in the NADPH-induced hydroxyl radical formation but not in that caused by ascorbate; however, lipid peroxidation induced by NADPH or ascorbate was suppressed by ethylenediaminetraacetate, indicating a crucial role of iron. Thus, the data indicate that the quinones destroyed CYP directly and not via oxygen activation or lipid peroxidation. — Environ Health Perspect 104(Suppl 6):1211–1218 (1996)

Key words: cytochrome P4502E1, humans, rats, benzene, hydroquinone, semiquinone, benzoquinone, OH· radicals, lipid peroxidation

Introduction

Cytochrome P450 (CYP EC 1.14.14.1) is a family of enzymes that oxidize or reduce chemicals to reactive intermediates that may alkylate nucleic acids, proteins, and CYP itself (*1*). These metabolites (epoxides, radicals, aldehydes, and quinones) are believed to be responsible for mutagenicity and carcinogenicity of chemicals, whereas their binding to CYP heme or protein inhibits or inactivates this enzyme.

Oxidative metabolites of benzene cause its myelotoxicity and carcinogenicity (*2*) and CYP destruction (*1,3*). Benzene treatment induces its own metabolism by CYP whereby it is oxidized to phenol, hydroquinone, and other metabolites by CYP2E1 in rat, mouse, and human (*3–11*). DT-diaphorase [NADP(H)-quinone acceptor oxidoreductase, EC 1.6.99.2] and ascorbate decreased metabolic activation of phenol to products that bind covalently to microsomal proteins, apparently because of inhibition of hydroquinone oxidation to the more reactive benzoquinone (*12*). It was suggested that OH· radicals were involved in the metabolism of benzene to genotoxic metabolites (*13*). The reactive oxygen species (ROS) formed by CYP monooxygenase apparently oxidizes benzene outside the CYP active site, as the reaction would not be inhibited by ROS scavengers otherwise (*14*).

Catechol, hydroquinone (HQ), and particularly its oxidized form, benzoquinone (BQ), exert their toxic effects on proteins, DNA, and CYP directly (*1,2,15*) or via oxygen activation (*16,17*). BQ reacts efficiently with thiol groups; its reaction with highly nucleophilic thiols of tubulin, which damages the mitotic spindle, appears to play a role in benzene myelotoxicity. BQ reacts spontaneously with DNA to form DNA adducts (*2*) and HQ indirectly damages DNA by forming reactive oxygen species, specifically hydrogen peroxide, which is involved in 8-hydroxydeoxyguanosine formation (*16*).

CYP destruction by HQ was suggested to be caused directly by its oxidized forms rather than via oxygen activation of CYP (*1*). In contrast, HQ and BQ appeared to protect CYP from destruction caused by radicals or ROS (*1*) that originated during the futile CYP cycle (*18–20*).

CYP2E1 was shown to yield very high levels of reactive oxygen species (*21*). HQ spontaneously reacts with oxygen forming semiquinone radical and superoxide anion radical; $^{\cdot}O_2$ may dismutate to H_2O_2 and these two oxygen species form hydroxyl (OH·) radicals (in the iron-catalyzed Haber-Weiss reaction), which are thought to be the most toxic reactive oxygen species to damage DNA (*17*).

The microsomal membrane system contains various oxidative and reductive enzymes, of which CYP and NADPH-dependent CYP reductase catalyze quinone oxidations and reductions and activate oxygen. Iron and quinones act similarly. Moreover, free radicals formed, as well as iron, may initiate lipid peroxidation. The question is, therefore, which of these harmful agents and reactions play major roles in CYP destruction *in vitro.*

Our previous results suggested that CYP destruction by HQ was caused by its conversion to BQ, whereas BQ acted directly, or partly, also via originating semiquinone radical (SQ) (*1*). The aim of the current

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Abbreviations used: BQ, benzoquinone; CYP, cytochrome P450; DEDTC, diethyldithiocarbamate; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; EDTA, ethyleneiamine-tetraacetic acid; ESR, electron spin resonance; HQ, hydroquinone; $^{\cdot}O_2^{-}$, superoxide anion radical; OH· radicals; ROS, reactive oxygen species; NADP(H), nicotinamide-adenine dinucleotide phosphate (reduced); TMP, 2,2,6,6-tetramethyl piperidine.

study was to investigate the interconversions among HQ, SQ, and BQ in liver microsomes to assess their roles in CYP destruction. The formation and persistence of SQ from HQ and BQ in spontaneous, NADPH, or enzyme-catalyzed reactions in microsomes were measured. We were interested in exploring whether CYP catalytic activity takes part in its destruction by the quinones. Estimation of the roles of SQ and BQ in CYP destruction could suggest the role played by SQ in other kinds of benzene toxicity.

Materials and Methods

Chemicals

All chemicals used were of analytical reagent grade. $MgCl_2$, KCl, Tris, NADP, NADPH, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, ascorbate, α-tocopherol, superoxide dismutase (EC 1.15.1.1.) (3900 U/mg), catalase (EC 1.11.1.6) (9740 U/mg), [U-^{14}C]-benzene (specific activity 121 mBq/mmol), and pentoxyresorufin were purchased from Sigma Chemical Co. (St Louis, MO). HQ and BQ were products of Aldrich (Milwaukee, WI) and were purified before use: HQ by crystallization, BQ by sublimation. Spin trap for $OH^{\cdot}$ radicals and superoxide anion radical determination, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), spin detector for superoxide anion radical determination 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron), and singlet oxygen detector 2,2,6,6-tetramethyl piperidine (TMP) were from Sigma–Aldridge production. Other chemicals were obtained from Lachema (Brno, Czech Republic).

Animals and Treatments

Adult male Wistar rats weighing 200 to 250 g (VELAZ, Prague, Czech Republic) were used. They were allowed to adapt at least a week after receipt. CYP2B1 was induced in rats by pretreatment with ip sodium phenobarbital, 80 mg/kg/day for 3 days. The animals were sacrificed 24 hr after the last dose. CYP2E1 was induced by benzene inhalation, 4 mg/liter of air, 20 hr/day for 3 days. In experiments aimed at estimating CYP destruction *in vivo*, rats were pretreated with phenobarbital in the same way and exposed to benzene inhalation for 12 hr (Figure 1).

Experiments with Human Liver Microsomes

Livers from human kidney transplantation donors were obtained from the Transplantation Centre of the Institute of Clinical and Experimental Medicine, Prague, Czech Republic, and their use was approved by the Ethical Committee of the Internal Grant Agency, Ministry of Health, Czech Republic.

Microsomes

Microsomes were prepared by differential centrifugation (*22*); protein was estimated with bovine serum albumin as standard (*23*).

Incubations

All incubations were carried out in glass-stoppered tubes (1-ml incubate) or Erlenmeyer flasks (2-ml incubate) using a shaking water bath at 37°C for 30 min (Tables 1,2; Figure 3), 60 min (Table 3; Figures 4,5,7–9), or 20 min (Figure 6). The incubation mixture contained microsomal protein at a final concentration of 1 mg/ml, NADPH-generating system (10 mM $MgCl_2$, 5 mM glucose 6-phosphate, 0.5 mM NADP, glucose 6-phosphate dehydrogenase, EC 1.1.1.49, 0.5 U/ml) and a KCl-Tris buffer, pH 7.4, final concentration of 150 mM KCl, and 50 mM Tris. In the incubations following NADPH oxidation by the quinones, NADPH was used instead of the NADPH-generating system. HQ or BQ solutions in distilled water were prepared just before use.

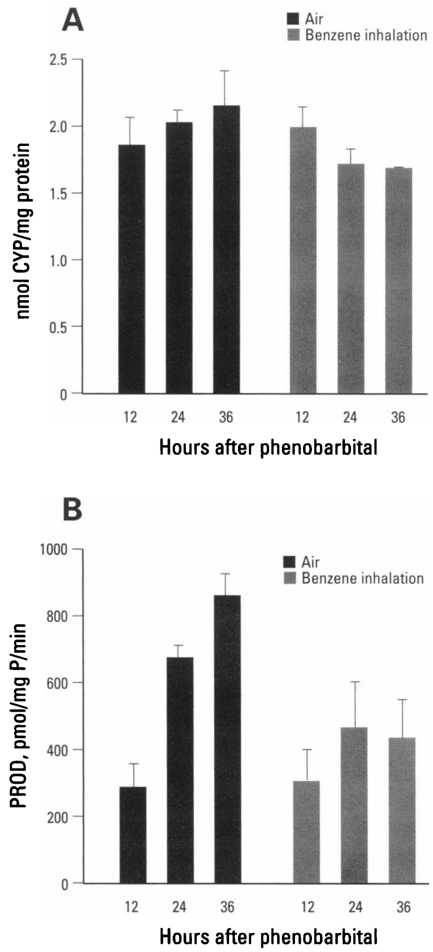

Figure 1. CYP destruction *in vivo* in rats pretreated with phenobarbital (80 mg/kg/day, ip for 3 days) to induce CYP 2B1 and subsequently exposed to 12-hr inhalation of benzene 12, 24, or 36 hr after the last dose of phenobarbital. (*A*) CYP level in microsomes; (*B*) pentoxyresorufin *O*-dealkylase activity.

Assays

Pentoxyresorufin *O*-dealkylase was assayed according to Lubet et al. (*24*). Soluble metabolites of [^{14}C]benzene were estimated according to Gut et al. (*10*) and products covalently bound to protein were assayed after extensively extracting the microsomes with alcohol/acetone/ethyl acetate (1:1:1) until the radioactivity of the extract reached background levels. [^{14}C]Benzene metabolites covalently bound to DNA in the incubates were estimated after phenol/chloroform extraction. The DNA concentration was estimated from the 260/280 nm ratio of DNA samples dissolved in distilled water after the extraction. Concentrations of HQ or BQ were assayed by ultraviolet–visible spectroscopy (UV–VIS) spectrophotometry (SPECORD 400, Carl Zeiss [Jena, Germany] after extraction into ethyl acetate by comparison with a calibration curve, essentially by methods described by Irons et al. (*25*).

The concentration of SQ radical was estimated by electron spin resonance (ESR). Samples were prepared either just before the measurement in the ESR room or in incubations of microsomes with the NADPH-generating system at specified temperature, in which the samples were frozen in liquid nitrogen just after incubation and transferred immediately to the ESR apparatus where the ESR measurement was performed. The total CYP content in microsomes before and after the incubation was estimated according to the method of Omura and Sato (*26*). The specific CYP immunochemical levels were those reported as before (*10*), and in human liver, microsomes were assayed in the same way (*4,5,21,27*).

Electron Spin Resonance Analysis

The ESR spectra were recorded on an ERS-220 spectrometer (Academy of Sciences, Berlin, Germany), magnetic field was measured on an H-NMR magnetometer (Radiopan, Poznan, Poland), microwave frequency on a frequency counter C3-54

(Union of Independent States) with the following conditions: microwave power 60 mW, modulation amplitude 0.02 mT, time constant 0.5 sec, scan speed 0.3 mT/min, calibration standard diphenyl-dipicryl-hydrazyl (g = 2.0037), temperature 25°C. ESR spectra were recorded as a first (in some cases as a second) derivation and main parameters as g-factor values; hyperfine coupling constant A, line width delta H_{pp} (peak-to-peak distance), and delta A_{pp} (peak-to-peak amplitude) were calculated according to Makino et al. (*28*). Analysis of oxygen radicals was made by using DMPO as a spin trap (*28*). Analysis of superoxide anion radicals was made using Tiron (1,2-dihydroxy-benzene-3,5-disulfonic acid, disodium salt) (*29,30*) as a specific spin detector to identify and determine superoxide anion radical. The spin trap DMPO was used to identify and determine $OH^{\bullet}$ radicals and superoxide anion radicals (*31*). It became obvious that the SQ radical formed a signal that prevented the measurement of the oxygen radical by ESR and another method was used instead to measure $OH^{\bullet}$ radicals; these were assayed by measuring formaldehyde formed from dimethyl sulfoxide as described (*32,33*). Lipid peroxidation was estimated according to Buege and Aust (*34*).

Results

Role of CYP in Benzene Oxidation to Soluble Metabolites and to Products that Bind Covalently to Proteins and DNA

In rats exposed to benzene, CYP2E1 induction resulted in a 5-fold increase in its immunochemical level (*10*) and increased microsomal benzene oxidation *in vitro*. Thus, induction resulted in rates of formation of soluble metabolites, DNA adducts, and protein adducts 8, 9, and 16 times more rapidly than controls at 1 mM benzene (Table 1). At 0.35 mM benzene, a higher proportion of metabolites was oxidized to protein-bound adducts than to soluble metabolites. CYP2B1 induction did not significantly increase the formation of soluble metabolites and DNA adducts, whereas protein-bound adducts were elevated 6- to 10-fold Figure 2A). The data support the view that CYP2E1 has higher affinity for benzene hydroxylation than CYP2B1 (*7*) and suggests that a significant part of DNA-binding metabolites may differ from protein-bound products. CYP2E1 inhibition by diethyldithiocarbamate (Table 2) revealed that both soluble and

Table 1. Effect of CYP2B1 or 2E1 induction on benzene oxidation to soluble metabolites and products covalently bound to proteins or DNA.

	Metabolites, nmol/mg p/min				Metabolites, nmol/mg DNA	
	0.35 mM Benzene		1.03 mM Benzene		1 mM Benzene	
Microsomes	Soluble	Protein-bound	Soluble	Protein-bound	DNA-bound	N^7-guanine
Control	0.701	0.053	1.297	0.077	0.21	ND
CYP 2B1	0.609	0.550	1.704	0.471	0.33	ND
CYP 2E1	3.301	2.271	10.767	1.267	2.00	44.1%

ND, no data. CYP2B1 was induced in rats pretreated with sodium ip, phenobarbital, 80 mg/kg/day for 3 days and sacrificed 24 hr after last dose. CYP2E1 was induced by benzene inhalation, 4 mg/liter of air, 20 hr/day for 3 days. The results are means of pooled microsomes from four to six rats.

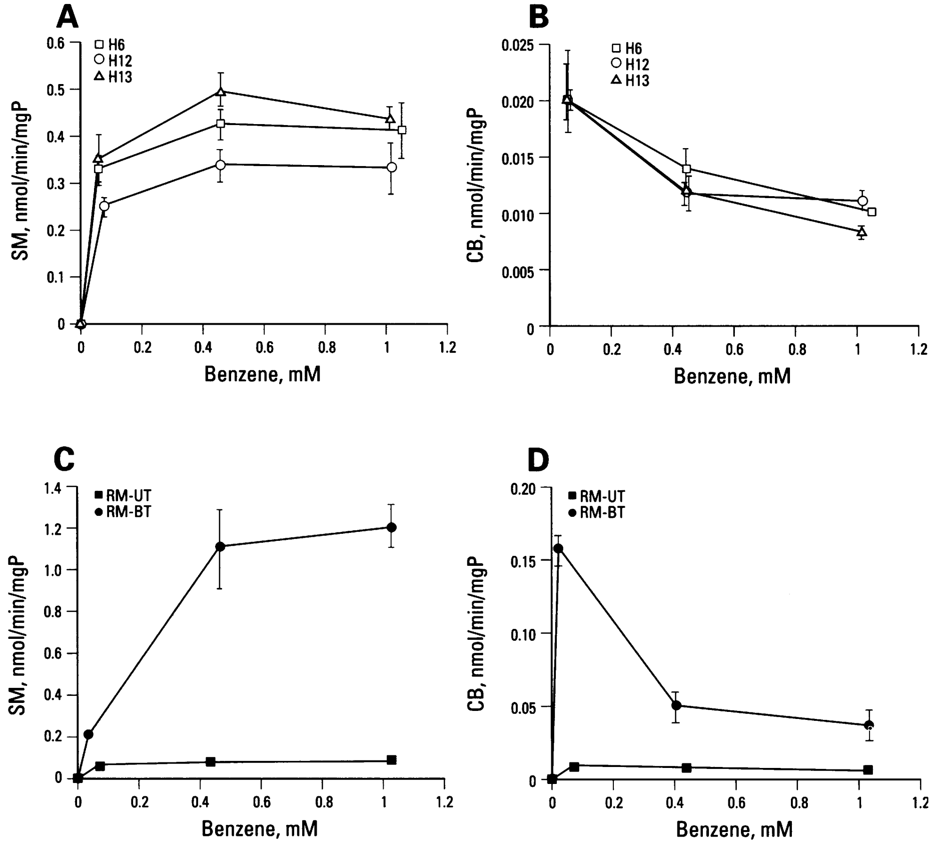

Figure 2. Benzene oxidation in various human liver microsomes to soluble metabolites (*A*) and covalently bound products (*B*) and in liver microsomes from control rats (RM-UT) or rats exposed to benzene (4 mg/liter 20 hr/day for 3 days) to induce CYP2E1 (RM-BT) microsomes, to soluble metabolites (*C*) and covalently bound products (*D*).

Table 2. Inhibition of benzene oxidation to soluble metabolites and products covalently bound to proteins in CYP2E1-induced liver microsomes (% of remaining activity).

Concentration of benzene, mM	Concentration of inhibitor, mM		Soluble metabolites, % of control	Protein adducts, % of control
0.3	None		100	100
	DEDTC	0.1	12	2
	DEDTC	0.3	2	2
2.6	None		100	100
	Ascorbate	1	105	25
	Tocopherol	1	118	99

DEDTC, diethyldithiocarbamate. CYP2E1 was induced by benzene inhalation, 4 mg/liter of air, 20 hr/day for 3 days. The results are means of pooled microsomes from four to six rats. Uninhibited metabolism at 0.3 mM benzene was (nmol/mg protein/min): soluble metabolites, 1.445 ± 0.134; protein adducts, 0.041 ± 0.005. At 2.6 mM benzene: soluble metabolites, 1.839 ± 0.147; protein adducts, 0.081 ± 0.033.

covalently bound metabolites are predominantly formed by CYP2E1. Ascorbate inhibited production of the covalently bound but not the soluble metabolites in agreement with the idea that the former are mostly the oxidation products of HQ. The lack of inhibition of the formation of covalently bound metabolites of benzene by α-tocopherol (Table 2) suggests that semiquinone (or other radicals) plays a minor role here. In agreement with these data, oxidation of benzene to soluble metabolites was proportional, while the formation of protein adducts was inversely related to benzene concentration (Figure 2) in CYP2E1-induced rat liver microsomes as well as in human liver microsomes. Benzene oxidation in human microsomes to both soluble ($r = 0.87$) and covalently bound ($r = 0.76$) metabolites significantly correlated with CYP2E1 immunochemical levels.

Nature of Toxic Oxidative Metabolites of Benzene

Induction of CYP2B1 in rats by phenobarbital, followed by subsequent inhalation of benzene (4 mg/liter, 12 hr) resulted in a significant, time-dependent destruction of CYP enzymes, especially CYP2B1 (Figure 1), suggesting that benzene metabolites could be responsible for CYP inactivation *in vivo*.

In vitro incubation of CYP2B1-induced rat liver microsomes with benzene or various benzene metabolites revealed that neither benzene nor phenol inactivated CYP enzymes, whereas significant, dose-dependent CYP inactivation was exerted by BQ > HQ > catechol. Investigation of the combined effect of these metabolites with a CYP cofactor, NADPH, revealed that catechol, HQ or BQ might also protect CYP against NADPH-mediated damage (Table 3).

The relative potencies of benzene metabolites to damage CYP and protect against NADPH-induced damage over a concentration range of 0.01 to 0.1 mM were catechol < HQ < BQ (Figure 3A–D). It was not obvious if these metabolites destroyed CYP directly or via oxygen activation or lipid peroxidation. Neither EDTA nor desferral reversed the toxicity of catechol or HQ, whereas the effect of BQ was slightly mitigated (Figure 4), suggesting a possible role of iron-mediated oxygen activation or lipid peroxidation in the quinone toxicity. Superoxide dismutase did not protect CYP against destruction by the quinones, but catalase partially reversed the catechol-induced CYP damage. Maintaining catechol or HQ reduced the damage, and

Table 3. CYP destruction by benzene and its metabolites *in vitro*.

	Incubation of liver microsomes		
	Without NADPH	With NADPH	
	CYP, % of	CYP concentration	
Addition, mM	basal concentration	No addition = 100%	NADPH = 100%
No addition	100 ± 7.2	100.0 ± 7.2	—
NADPH	43.8 ± 3.0*	43.8 ± 3.0*	100.0 ± 6.8
Benzene	108.9 ± 2.4	56.3 ± 0.9*	128.5± 2.1**
Phenol	110.4 ± 7.0	91.8 ± 1.8*	209.6 ± 4.1**
Catechol	86.3 ± 4.5*	62.1 ± 9.4*	141.8 ± 21.5**
Hydroquinone	53.4 ± 11.3*	59.3 ± 9.4*	135.4 ± 21.5**
Benzoquinone	31.6 ± 4.9*	58.5 ± 6.4*	133.6 ± 14.6**

Results are means ± standard deviation of at least four separate samples. Liver microsomes from phenobarbital-induced rats were incubated for 60 min at 37°C in air, with no addition, with NADPH-generating system (1 mM NADP), or with a 1 mM concentration of benzene or its metabolites or their combination with NADPH. Significant at $p < 0.05$ from no addition (*) or from NADPH (**).

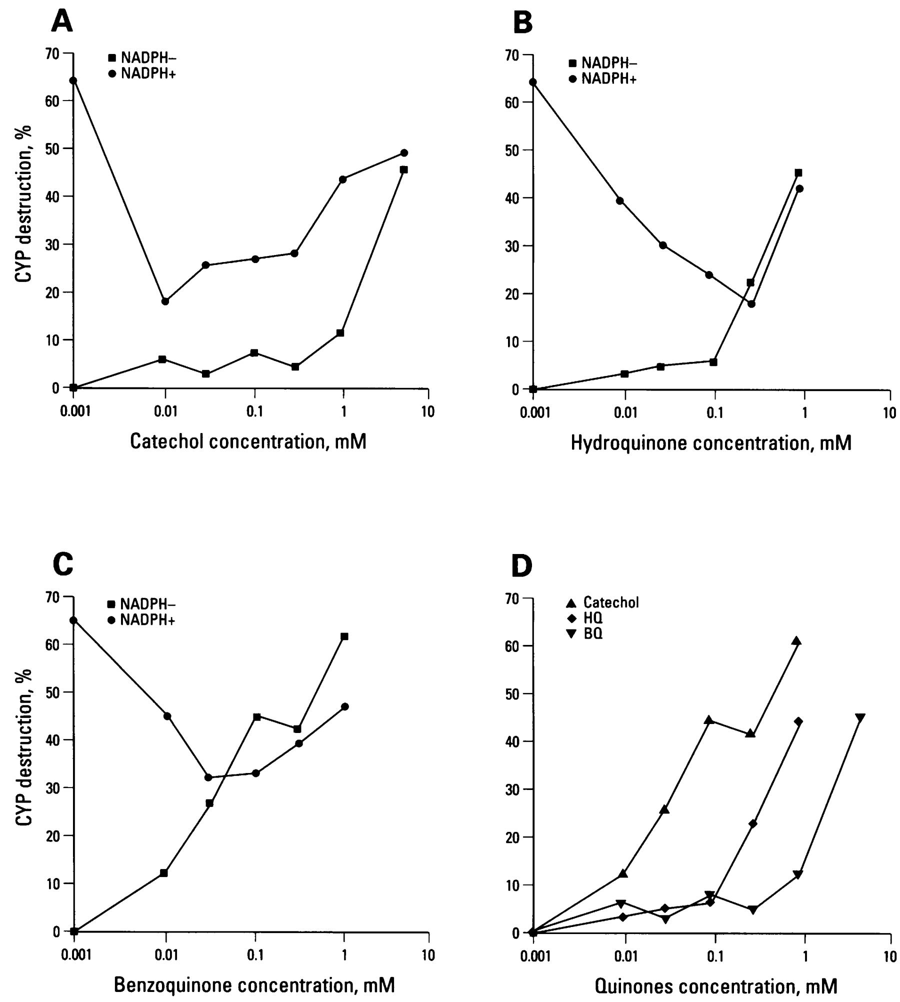

Figure 3. CYP destruction *in vitro* after incubation of liver microsomes from phenobarbital-pretreated rats (Figure 1) with various concentrations of catechol (*A*), HQ or (*B*), BQ (*C*) with (NADPH+) or without NADPH-generating system (NADPH–). (*D*) Comparison of the effects of three quinones incubated with microsomes in the absence of NADPH-generating system.

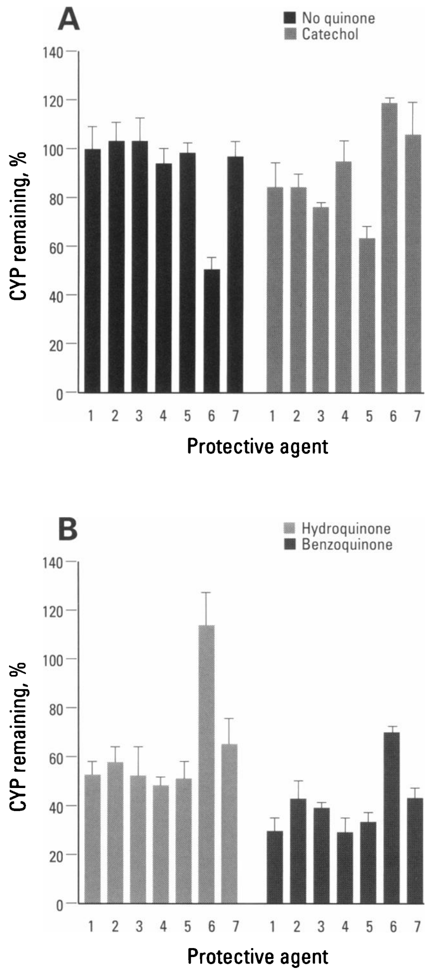

Figure 4. CYP destruction *in vitro* after the incubation of liver microsomes from rats pretreated with phenobarbital (Figure 1). Incubation (*A*) without or with 1 mM catechol, (*B*) with HQ or BQ. Effect of protective agents: (1) none; (2) 1 mM EDTA; (3) 1 mM desferral; (4) superoxide dismutase, 5 µg/ml; (5) catalase, 25 µg/ml; (6) ascorbate, 1 mM; (7) α-tocopherol, 1 mM.

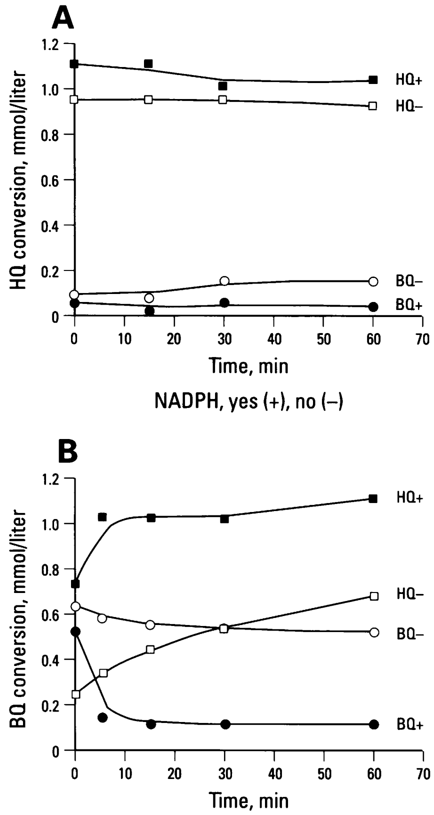

Figure 5. *A*, The level of HQ and the formation of BQ from 1 mM HQ incubated in liver microsomes from phenobarbital-pretreated rats (Figure 1) without (HG–) or with the NADPH-generating system (HG+) and with (*B*) the level of BQ and the formation of HQ from 1 mM BQ incubated without (BQ–) or with the NADPH-generating system (BQ+).

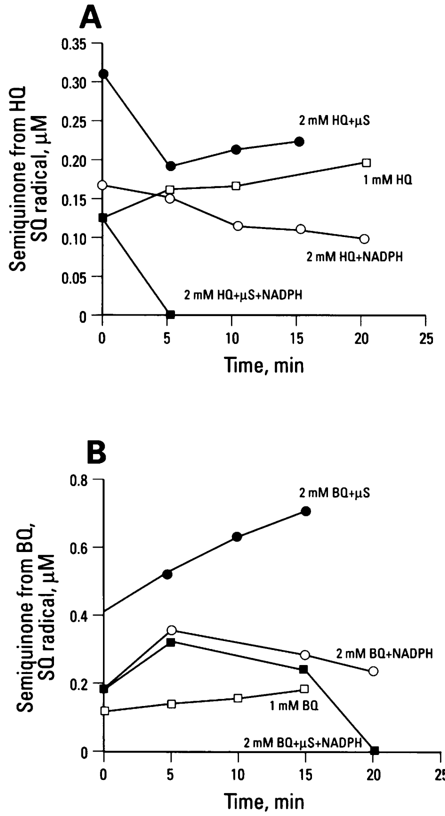

Figure 6. (*A*), The formation of SQ from HQ and (*B*), the formation of SQ from BQ, BQ incubated at 25°C for 20 min in 1 mM solutions in 1.15% KCl, 50 mM HCl-Tris buffer, pH 7.4, without (1 mM HQ, 1 mM BQ) or with the NADPH-generating system (2 mM HQ + NADPH or 2 mM BQ + NADPH), or in 2 mM solutions with the same liver microsomes (µS) without or with the NADPH-generating system.

reduction of BQ by ascorbate was the most effective protective means against their damaging effect on CYP enzymes and increased CYP levels in microsomes (Figure 4). The SQ radical trapping agent, α-tocopherol, offered a small but significant protection of CYP against HQ and BQ.

NADPH-mediated and quinone-induced CYP damage (Table 3) were mutually decreased in their joint action. Since BQ effectively oxidized NADPH (data not shown), while NADPH reduced BQ (Figure 5), CYP may have been protected by their mutual interaction. Obviously NADPH prevented HQ oxidation to BQ in liver microsomes (Figure 5). Even the microsomes without added NADPH showed some potency to reduce the quinones (Figure 5, lower part).

Since the partial CYP protection by α-tocopherol (Figure 4) suggested that the SQ radical could play a role in BQ or HQ toxicity, its formation in microsomes was monitored by ESR (Figure 6). In agreement with the ability of liver microsomes to reduce BQ (Figure 5), a spontaneous formation of SQ radical from HQ was inhibited by added microsomes. The NADPH-generating system without the microsomes suppressed the formation of SQ, while microsomes with the NADPH-generating system quenched SQ so rapidly that it was measurable at the start of incubation but absent after a 5 min incubation. The formation of SQ radical from BQ (which was more rapid in BQ solution in buffer than in HQ solution; data not shown) did not seem to be effectively quenched by microsomes alone. The NADPH-generating system alone or with microsomes reduced SQ formation.

The possibility that reactive oxygen species were involved in the toxicity of quinones was studied in microsomes from animals in which CYP 2B1 or 2E1 had been induced. Neither HQ nor BQ induced $OH^{\cdot}$ radical formation in the microsomes. The NADPH-mediated formation of $OH^{\cdot}$ radicals was increased by HQ or BQ in proportion to their concentrations (Figure 7). The quinones and NADPH mutually decreased the CYP destruction they produced acting separately, i.e., the quinones protected CYP from destruction caused by NADPH and NADPH protected CYP from destruction caused by the quinones. Effects were similar in all three kinds of microsomes.

Ascorbate (1 mM) stimulated the formation of $OH^{\cdot}$ radicals (Figure 8) and produced significant CYP destruction. Neither

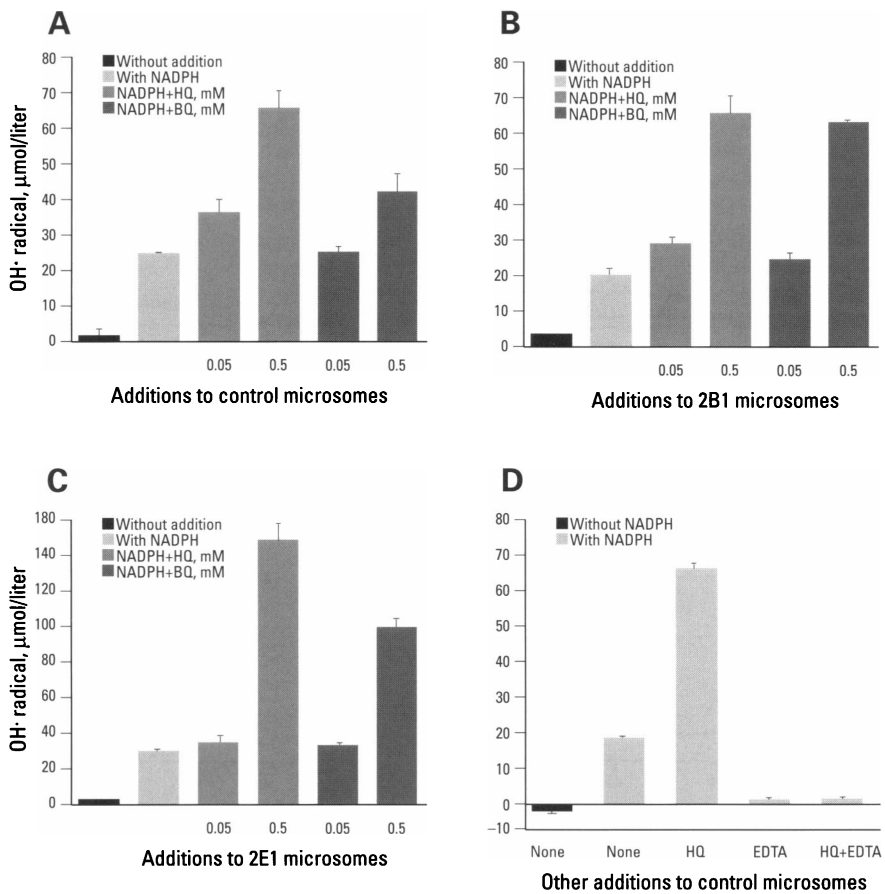

Figure 7. OH• radical generation in rat liver microsomes from controls (*A*), phenobarbital (*B*), or benzene-pretreated (*C*) animals (explained in the legends for Figures 1 and 2). The background level of OH• radical generation was enhanced by the NADPH-generating system (NADPH) without or with HQ or BQ added at 0.05 or 0.5 mM concentration and inhibited by 0.1 mM EDTA in control (*D*) and similarly in all three types of microsomes.

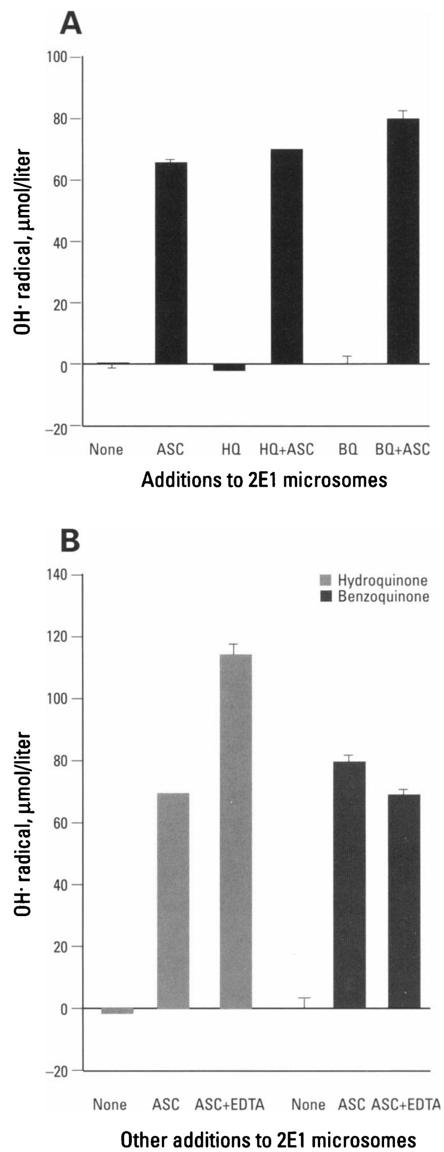

Figure 8. OH· radical generation in liver microsomes from rats exposed to benzene to induce CYP2E1 (2E1 microsomes). (*A*) The microsomes were incubated at 37°C for 60 min without or with 1 mM ascorbate (ASC), 0.5 mM HQ or 0.5 mM BQ or their combinations. (*B*) The microsomes were incubated with hydroquinone or benzoquinone and "other additions": none, with 1 mM ASC or ASC with 0.1 mM EDTA.

HQ nor BQ decreased the ascorbate-induced OH• radical formation. However, the quinones and ascorbate mutually suppressed the damage they induced to CYP while acting separately. Chelation of iron by EDTA decreased the formation of OH• radicals by NADPH or NADPH + quinones (Figure 7, lower right), but EDTA did not suppress the ascorbate-induced OH• radicals (Figure 8).

We also investigated a possible role of lipid peroxidation in CYP destruction in CYP2E1-induced microsomes where OH• radical formation and lipid peroxidation were more effective than in the control or 2B1-induced microsomes. Neither HQ nor BQ induced lipid peroxidation (Figure 9), although they caused CYP destruction (Table 3; Figure 3). NADPH induced a significant level of lipid peroxidation in liver microsomes, which was decreased by both HQ and BQ in proportion to their concentrations (Figure 9B).

Discussion

Quinone metabolites of benzene, particularly BQ, which damage both proteins and DNA, are among the most toxic metabolites of benzene (*2*). We have reported that *in vivo* and *in vitro* CYP destruction caused by benzene and its metabolites was related to BQ and possibly to the SQ radical rather than to HQ or catechol (*1*). However, autooxidation of various quinones is accompanied by the formation of reactive oxygen species (*17*). Moreover, radicals induce lipid peroxidation and SQ is a product of redox cycling of HQ/BQ. We have, therefore, investigated the role of reactive oxygen species and lipid peroxidation in the CYP destruction caused by quinone metabolites of benzene.

Lack of CYP protection by superoxide dismutase indicated superoxide anion radical did not play a marked role in quinone toxicity, but partial reversal of catechol-induced CYP damage suggested some role of hydrogen peroxide or subsequently formed OH• radicals in its toxicity.

Ascorbate was the most effective protective agent against catechol, HQ, and BQ, indicating that their reduced forms were not toxic to CYP. On the contrary, significantly increased CYP concentrations in catechol + ascorbate and hydroquinone + ascorbate-treated microsomes suggested a CYP activation that increased its binding

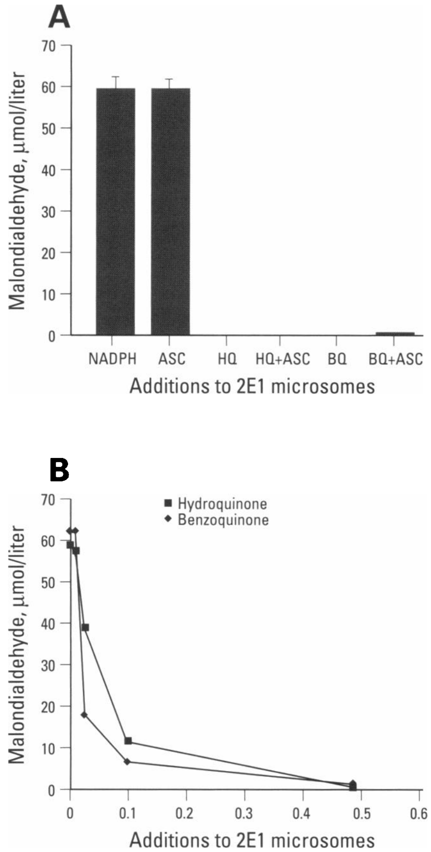

Figure 9. Lipid peroxidation estimated as malondialdehyde formation (µmol/liter) in liver microsomes from rats exposed to benzene to induce CYP2E1 (2E1 microsomes). (*A*) Effects of NADPH-generating system, 1 mM ascorbate, 0.5 mM HQ, 0.5 mM BQ, or their combined actions. (*B*) Inhibition of the NADPH-induced lipid peroxidation by increasing concentrations of HQ or BQ.

of carbon monoxide. A small, but significant protection of CYP against HQ and BQ by α-tocopherol indicated a possible role of SQ in their toxicity. The data indicate that the quinones damage CYP via their oxidative forms, i.e., that HQ is rather toxic via its SQ or BQ product, while reactive oxygen species or lipid peroxidation might have additional, but less important, toxic effects.

HQ is known to autooxidize to SQ and BQ with simultaneous formation of a superoxide anion (*17*) and hydrogen peroxide (*16*). Iron, which is present *in vivo* as well as in phosphate buffers, catalyzes Haber-Weiss and Fenton reactions that form hydroxyl radicals. However, OH• radicals appeared to play a negligible role in HQ- or BQ-induced CYP destruction, since their formation was not stimulated by HQ or BQ under conditions in which either quinone caused marked CYP destruction.

The addition of EDTA reduced both hydroxyl radicals and CYP destruction, suggesting a causal relationship. Nevertheless, the combined action of NADPH and HQ or BQ resulted in less CYP destruction when compared with their separate actions, whereas the formation of OH• radicals was markedly higher. Thus, the data suggest that the joint effect of NADPH and the quinones on CYP is not significantly mediated via OH• radicals.

Iron played an important role in the formation of OH• radicals induced by NADPH alone and acting together with HQ or BQ, as indicated by the decrease in hydroxyl radical formation caused by EDTA. The mutual suppression of CYP damage caused by NADPH and the quinones during their joint action could be due to NADPH oxidation by HQ or BQ and by reduction of the quinones by NADPH. Both HQ and BQ oxidized NADPH added to microsomes or in buffer, indicating a significant role of chemical NADPH oxidation. At the same time, NADPH prevented spontaneous oxidation of HQ to BQ so that HQ levels were higher and BQ concentrations were lower than in microsomes without NADPH. In the same way, BQ was reduced in NADPH-supplemented rat-liver microsomes, as indicated by the rapid appearance of HQ and disappearance of BQ. The absence of any EDTA effect on the ascorbate, ascorbate + hydroquinone or ascorbate + benzoquinone-induced OH• radical formation suggests that iron did not play an important role in the ascorbate effect.

Lipid peroxidation did not mediate CYP destruction by the quinones, since they did not induce it, but it could play a significant role in the NADPH-induced CYP destruction, as has been reported previously (*18,19*). Unlike hydroxyl radicals, increasing concentrations of the quinones proportionally decreased the NADPH-induced lipid peroxidation. Since low concentrations of the quinones decreased CYP destruction induced by NADPH (Figure 3), the quinones may have partly protected CYP against destruction by decreasing lipid peroxidation. However, at quinone concentrations above 0.5 mM, their direct destructive effects apparently prevailed, since they completely suppressed lipid peroxidation; yet they markedly destroyed CYP.

The present results confirm previous indirect evidence (*1*) that BQ and SQ are responsible for the CYP destruction caused by HQ and BQ and show that neither OH• radicals nor lipid peroxidation play a significant role in the quinone toxicity. Ascorbate probably caused CYP destruction by inducing high levels of OH• radicals and lipid peroxidation, while iron seemed to play no role in its action. The mutual mitigating effect of the quinones, NADPH, and ascorbate on CYP destruction they caused while acting separately appeared to be mostly related to their chemical interactions. Iron apparently played a key role in the NADPH-induced lipid peroxidation that EDTA completely inhibited. The fact that HQ and BQ also inhibited lipid peroxidation might be related to the observation that HQ and 1,2,4-benzenetriol chelate iron (*35*), which may explain their interactions in causing and reversing CYP destruction. It also indicates that several mechanisms of toxicity may participate in these complex mixtures, while in specific conditions one of them predominates.

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